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PROTEOSE-PEPTONE OF MILK IN RELATION
TO OTHER MILK PROTEINS

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517. SIALIC ACID AND HEXOSE CONTENTS OF PROTEOSE-PEPTONE OF MILK IN RELATION TO OTHER MILK PROTEINS*

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Endeavours at chemical elucidation of the mechanism of rennet action on casein have led to the recognition of the release of a glycopeptide from casein characterised by the presence of sialic acid (Alais & Jolles, 1961; Beeby, 1963; Jolles *et al.*, 1961; Koning *et al.*, 1963; Lindqvist, 1963). Subsequently, increasing attention is at present focussed on sialic acid in relation to its distribution and role in milk proteins (Gupta & Ganguli, 1965 *a*, 1965 *b*). The existence of sialic acid in whole casein (Lindqvist, 1963) and in *a* and *k*-caseins (Gupta & Ganguli, 1965 *a*; Marier *et al.*, 1963; Beeby, 1963; Thompson & Pepper, 1962) has also been well documented by several workers.

Of recent interest is the report by Brunner & Thompson (1959) and Marier *et al.* (1963) that proteose-peptone, a minor protein fraction of milk, is observed to be considerably rich in sialic acid. Furthermore, the resemblance in the chemical entities present in the glycopeptide released by rennet from casein (Brunner & Thompson, 1959) and in proteose-peptone (Thompson & Brunner, 1959) fractions of milk have further motivated us to assess the quantitative significance of such sugar moiety in milk proteins. A logical approach was, therefore, to evaluate the relative contents of sialic acid in proteose-peptone and other milk proteins like casein and whey proteins. Since sialic acid is normally bound to protein molecule through a glycosidic linkage involving hexose (Lindqvist, 1963; Winzler, 1955; Jolles *et al.*, 1964), proteose-peptone of milk was also analysed for its hexose content. It is the purpose of the present paper to document these observations along with a comparative picture on similar data from cow and buffalo milks.

EXPERIMENTAL

Materials

Milk samples used were from the Institute's herd. NANA and 2-thiobarbituric acid were

gift samples from Sigma Chemical Company, U.S.A.

Isolation of Proteose-Peptone and Proteose from Milk

Proteose-peptone and proteose were isolated from milk by the procedure of Ganguli *et al.* (1965). Casein and whey proteins were first separated by adjusting the pH of boiled milk to 4.5. The filtrate was then used for isolation of proteose-peptone by trichloroacetic acid and proteose by ammonium sulphate precipitation. The precipitates thus obtained were dialysed and dried to powder in a vacuum desiccator.

Fractionation of Milk Proteins

(a) *Precipitation of Casein*—7 ml of milk was adjusted to pH 4.5 with 1 N sulphuric acid. The precipitated casein was washed four times with acetate buffer, pH 4.5 using 5 ml at a time. Finally this precipitate was used for the estimation of sialic acid in the casein fraction.

(b) *Precipitation of Casein plus Whey Proteins*.—7 ml of milk was immersed in a boiling water-bath for 15 min. to precipitate whey proteins by heat denaturation and the pH of the milk was then adjusted to 4.5. The precipitated casein and whey proteins were washed with acetate buffer as done above.

(c) *Precipitation of Casein, Whey Proteins and Proteose-Peptone*.—Another 7 ml aliquot of the same milk sample was treated with 1 ml of 80% trichloroacetic acid. The precipitated proteins (casein + whey proteins + proteose-peptone) were then washed with acetate buffer as indicated above.

The proteins obtained in (a), (b) and (c) were then hydrolysed separately with sulphuric acid at 80° for 45 min. according to Marier *et al.* (1963). The pH was then brought back to 4.5 with 0.1 N NaOH and centrifuged at 2800 r.p.m. for 5 min. The supernatant was transferred to a test-tube. The residue was washed once with acetate buffer, pH 4.5, centrifuged and washings added to the previous supernatant and volume noted. 0.1 ml of chloroform was added for clarification. 0.2 ml

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Sialic Acid and Hexose Contents of Proteose-Peptide of Milk

of such solution was then subjected to sialic acid analysis.

Determination of Sialic Acid in Milk Proteins

(i) *In Proteose-Peptide and Proteose Samples.*—20–30 mg. of either proteose-peptide or proteose was moistened with 4.5 ml of distilled water and hydrolysed with 0.5 ml of 1 N sulphuric acid for 45 min. at 80° C. The hydrolysate was cooled and pH adjusted to 4.5 by the addition of 0.45 ml of 0.1 N NaOH. This hydrolysate was subjected to thiobarbituric acid assay for the estimation of sialic acid.

(ii) *Assay of Sialic Acid.*—N-acetyl neuraminic acid (NANA) was used as a standard for expressing the sialic acid content in milk protein fractions. Estimation was carried out according to the thiobarbituric acid assay described earlier by Gupta & Ganguli (1965*a*). Colour was read at 549 mμ using cell of 1 cm light path and a molecular weight of 309 for NANA (Warren, 1959, 1960) was used for calculations.

(iii) *In Fractionated Milk Proteins.*—The sialic acid present in different protein fractions as obtained by fractionation of milk indicated above, was calculated as follows:

Casein sialic acid = Sialic acid value of (a)
Whey proteins sialic acid = Sialic acid value of (b) — Sialic acid value of (a).
Proteose-peptide sialic acid = Sialic acid in (c) — Sialic acid in (b).

Paper Chromatography of NANA

The samples for the detection of NANA were spotted on Whatman No. 1 filter-paper and chromatographed by ascending method using ethanol : water : conc. ammonia (80 : 20 : 1) as the developing solvent (Warren, 1960). The paper was sprayed with an orcinol-TCA reagent (Gupta & Ganguli, 1965*a*).

Analysis of Hexose

The protein-bound hexose in both proteose-peptide and proteose was evaluated by a method described by Winzler (1955) using orcinol-sulphuric acid reagent for developing the colour. About 10 mg of weighed material was dissolved in 1 ml of 0.1 N NaOH. 0.5 ml of orcinol-sulphuric acid reagent was added and mixed well. The mixture was then heated in a water-bath at 80° C for exactly 15 min. after which the samples were cooled in tap water. The developed colour was finally read at 540 mμ. Calculations were based on a standard curve prepared similarly using a mixture of galactose and mannose (0.1 mg/ml each).

RESULTS AND DISCUSSION

Concentration of Sialic Acid in Proteose and Proteose-Peptide of Milk.—The isolated dry samples of proteose and proteose-peptide were analysed for their sialic acid content and results are expressed in Table 1. It is apparent from

TABLE 1. *Sialic Acid Contents of Proteose and Proteose-Peptide Isolated from Milk of the Cow and the Buffalo*

Species	Breed	Nature of sample	No. of sample analysed	Sialic acid (mg/g)		Average of species	
				Range	Average	Proteose	Proteose-peptide
Cow	Red Sindhi	Proteose	7	30.1–50.6	36.5	33.6	24.1
		Proteose-peptide	7	24.9–28.7	26.0		
		Casein*	10	3.9–6.8	5.5		
	Sahiwal	Proteose	8	21.9–42.1	31.9		
		Proteose-peptide	8	16.6–32.8	20.9		
		Casein*	10	4.3–6.7	5.6		
	Tharparkar	Proteose	6	23.2–44.2	32.6		
		Proteose-peptide	7	16.0–38.4	25.4		
		Casein*	10	3.8–7.6	5.7		
Buffalo	Murrah	Proteose	15	7.4–17.2	11.5	11.5	9.6
		Proteose-peptide	15	5.6–11.2	9.6		
		Casein*	10	1.7–3.2	2.5		

* Values from Gupta & Ganguli (1965*a*).

the table that proteose samples have always a higher concentration of sialic acid than the corresponding proteose-peptone samples, the average values being 33.6 mg/g and 24.1 mg/g for cow milk and 11.5 mg/g and 9.6 mg/g for buffalo milk, respectively. These results show that proteose-peptone fraction from milk has almost a four-fold concentration of sialic acid compared to its concentration in casein fraction (Gupta & Ganguli, 1965a; Lindqvist, 1963). Furthermore, similar data on proteose-peptone and proteose from buffalo milk samples have exhibited a lower value than the cow milk samples. The observed values on sialic acid of proteose-peptone from the cow milk appeared to be higher compared to reported values of Marier *et al.* (1963).

Sialic Acid Content of Milk Proteins

(a) *Sialic Acid in Total Milk Proteins.*—Estimation was next carried out to note the sialic acid concentration in total milk proteins. The TCA precipitable proteins were collected as done in method (c) of fractionation of milk protein procedure and sialic acid estimated as described above. Data are presented in Table 2. Milk proteins from the cow indicated an almost double concentration of sialic acid, 24.42 mg/100 ml milk, compared to buffalo milk proteins, 12.40 mg/100 ml. Amongst the three breeds of cow, Sahiwal showed slightly higher concentration of sialic acid, 27.20 mg/100 ml, than Tharparkar and Red Sindhi breeds.

TABLE 2. *Sialic Acid Contents of Total Milk Proteins*

Species	Breed	No. of samples analysed	Sialic acid (mg./100 ml. milk)	
			Average values for breeds	Average values for species
Cow	Red Sindhi	10	23.05	..
	Sahiwal	10	27.20	24.42
	Tharparkar	10	23.02	..
Buffalo	Murrah	10	12.40	12.40

(b) *Distribution of Sialic Acid in Different Protein Fractions of Milk.*—The sialic acid concentration was determined in different milk proteins such as casein, whey proteins and proteose-peptone obtained by fractionating the same milk sample. These results are expressed in Tables 3, 4, 5 and 6 alongwith the analysis of variance. It is clear from the results that casein fraction of milk contributes a maximum share, to the extent of 17.95–19.75 mg (Table 3) of the total sialic acid of 24.42 (Table 2) present in 100 ml of cow milk. The buffalo milk casein also indicates maximum sialic acid content, 9.05 mg (Table 3) out of a total of 12.40 mg/100 ml milk (Table 2).

TABLE 3. *Sialic Acid Content of Casein from Milk of the Cow and the Buffalo*

Species	Breed	No. of samples analysed	Sialic acid (mg/100 ml milk)	
			Range	Mean \pm Standard error
Cow	Red Sindhi	10	13.42–26.56	18.43 \pm 1.457
	Sahiwal	10	14.39–28.92	19.75 \pm 2.275
	Tharparkar	10	12.87–24.77	17.95 \pm 1.131
Buffalo	Murrah	10	5.53–11.76	9.05 \pm 0.700

Analysis of Variance

Variation due to	d.f.	M.S.S.	F. value	F. at 1% level
Between three breeds of cows	2	9.217	0.382	..
Between cows and buffaloes ..	1	812.703	33.72	7.31
Error ..	39	24.101

Of the other two protein fractions, 3.28 mg and 2.49 mg were due to proteose-peptone (Tables 4, 6) and whey proteins (Tables 5, 6), respectively, in cow milk samples whereas the corresponding values for buffalo milk were 1.75 mg and 1.60 mg (Table 6). Such lower values for sialic acid in buffalo milk protein fractions were proved to be statistically significant at 1% level.

TABLE 4. *Sialic Acid Content of Proteose-Peptone of Milk from the Cow and the Buffalo*

Species	Breed	No. of samples analysed	Sialic acid (mg/100 ml milk)	
			Range	Mean \pm Standard error
Cow	Red Sindhi	10	1.53-4.57	2.28 \pm 0.499
	Sahiwal	10	3.60-5.12	4.31 \pm 0.359
	Tharparkar	10	2.07-4.99	3.25 \pm 0.352
Buffalo	Murrah	10	1.11-2.91	1.78 \pm 0.290

Analysis of Variance

Variation due to	d.f.	M.S.S.	F. value	F. at 1% level
Between three breeds of cows	2	10.840	9.071	...
Between cows and buffaloes	1	28.057	23.480	7.31
Error	39	1.195		..

TABLE 5. *Sialic Acid Content of Whey Proteins of Milk of the Cow and the Buffalo*

Species	Breed	No. of samples analysed	Sialic acid (mg/100 ml milk)	
			Range	Mean \pm Standard error
Cow	Red Sindhi	10	1.11-3.18	2.34 \pm 0.377
	Sahiwal	10	2.07-4.15	3.32 \pm 0.483
	Tharparkar	10	0.93-2.63	1.82 \pm 0.355
Buffalo	Murrah	10	0.69-2.63	1.52 \pm 0.090

Analysis of Variance

Variation due to	d.f.	M.S.S.	F. value	F. at 1% level
Between three breeds of cows	2	6.1438	4.708	..
Between cows and buffaloes ..	1	14.1934	10.877	7.31
Error ..	39	1.3049

TABLE 6. *Distribution of Sialic Acid in Different Protein Fractions of Milk from the Cow and the Buffalo*

Species	Breed	Sialic acid (mg/100 ml milk)						
		Total	Ave.	Casein	Ave.	When proteins	Ave.	Proteose-peptone Ave.
Cow	Red Sindhi	23.05	..	18.43	..	2.34	..	2.28 ..
	Sahiwal	27.20	24.42	19.57	18.65	3.32	2.49	4.31 3.28
	Tharparkar	23.02	..	17.95	..	1.82	..	3.25 ..
Buffalo	Murrah	12.40	..	9.05	..	1.60	..	1.75 ..

Considering the contribution in sialic acid content by the protein fractions, it can be seen that sialic acid due to casein was between 71 to 80%, due to proteose-peptone 10-15% and whey proteins have contributed 8-12% of sialic acid.

On the other hand, an evaluation of the content of sialic acid in these protein components in milk revealed that proteose-peptone was the richest fraction having 24.1 mg/g (Table 1) and next was casein. Whey proteins seem to have the lowest content of sialic acid.

The notable outcome of the present study which deserves special attention was the lower values of sialic acid in all fractions of proteins from buffalo milk compared to cow milk. With a lower value of sialic acid in α -casein, k -casein (Gupta & Ganguli, 1965 *a*) and in proteose-peptone (Table 1), one would expect a differential pattern in the rate of rennet action which involved the release of sialic acid from k -casein (Gupta & Ganguli, 1965 *b*; Lindqvist, 1963) and proteose (Ganguli *et al.*, 1966 *a*) by rennet.

Isolation and Characterization of Sialic Acid from Proteose and Proteose-Peptone of Milk

After establishing the presence of sialic acid in proteose and proteose-peptone, it was next thought worthwhile to characterize sialic acid present in these samples since it represents a group of derivatives of neuraminic acid (Gottschalk, 1960). Procedure used for casein sialic acid identification was adopted. 200 mg of proteose-peptone was hydrolysed with 5 ml of 0.1 N sulphuric acid at 80°C for 45 min. The supernatant was subjected to ion-exchange chromatography on a Dowex-1-formate column of 8 cm. \times 1.2 cm size. Sialic acid was then eluted from the column with 50 ml of 0.6 M formic acid and the eluate evaporated to dryness in a vacuum oven at a temperature not exceed-

ing 60°C. The concentrated material was dissolved in small volume of water and subjected to paper chromatography as indicated above. Side by side an authentic sample of NANA was also spotted and developed on the same chromatogram. All proteose-peptone samples treated in this manner gave a positive test for sialic acid and had R_f value identical with that of NANA. It appeared, therefore, that the same derivative of neuraminic acid which was present in k -casein (Lindqvist, 1963) was present in proteose-peptone. Based on such similarity, one can logically correlate these two fractions of milk in their appearance as products of mammary origin.

Hexose Content of Proteose and Proteose-Peptone from Cow and Buffalo Milk

(a) *Concentration of Hexose.*—The bound hexose content of proteose-peptone and proteose samples isolated from milk was evaluated by the orcinol-sulphuric acid method. These results are expressed in Table 7. Proteose samples appear to contain more hexose than the corresponding proteose-peptone samples. Cow milk fractions have considerably higher content of hexose in both proteose and proteose-peptone, 2.78% and 2.30% respectively, than buffalo samples which have 1.09% and 0.80% for proteose and proteose-peptone, respectively. The data on cow milk samples concur with the reported values of Brunner and Thompson (1959). The lower values of hexose in proteose-peptone than proteose indicates that hexose moiety was probably present in the proteose part of proteose-peptone. Such contention was further supported by the fact that peptone was a sialic acid negative fraction as revealed by gel filtration on Sephadex G-50 (Ganguli *et al.*, 1966 *b*). The lower content of hexose in buffalo samples was expected from the lower content of sialic acid for buffalo proteose and proteose-peptone samples (Table 1).

TABLE 7. *Concentration of Bound Hexose in Proteose and Proteose-Peptide of Milk from the Cow and the Buffalo*

Species	Breed	Nature of sample	Bound hexose (%)			
			Range	Average	Average for species	
					Proteose	Proteose-peptide
Cow	Red Sindhi	Proteose (6)*	1.90-4.55	3.09		
		Proteose-peptide (6)*	2.20-4.52	3.06		
	Sahiwal	Proteose (6)	1.61-4.32	2.70		
		Proteose-peptide (6)	1.13-3.60	1.94	2.78	2.30
	Tharparkar	Proteose (6)	2.24-2.91	2.55		
		Proteose-peptide (6)	1.53-2.39	1.92		
Buffalo	Murrah	Proteose (10)	0.69-1.57	1.09		
		Proteose-peptide (10)	0.66-0.97	0.80	1.09	0.80

* Figures under parentheses refer to the number of samples analysed.

(b) *Characterization of Bound Hexose.*— Attempt was next made to identify and characterize the hexose component present in proteose-peptide. For such purpose, the method described by Thomson & Brunner (1959) was followed with suitable modification. Samples containing 125 mg either proteose or proteose-peptide were hydrolysed in 5 ml of 2 N HCl at 100° for 3 hours. The hydrolysates were dialysed against several changes (at least four times) of distilled water which were combined, evaporated to dryness and extracted with pyridine. Such extracts were then chromatographed on paper using amyl alcohol: pyridine: water (4:3:2) as solvent by descending method (Thompson & Brunner, 1959). The dried paper was sprayed with aniline phthalate for detecting sugars (Partridge, 1949).

The hydrolysate from both proteose and proteose-peptide gave positive spots for glucose, galactose and glucosamine on the paper chromatogram and thereby indicate the presence of these hexose molecules in the samples analysed. Cow and buffalo samples exhibited similar results in this respect. These results again indicate a similarity in the composition of casein (Reynolds *et al.*, 1959) and the glycopeptide released from casein by rennet (Thompson &

Brunner, 1959) with proteose-peptide in their hexoses engaged in linking sialic acid with the peptide chain.

SUMMARY

1. The sialic acid content of proteose and proteose-peptide isolated from the milk of cow was evaluated to be 33.6 mg/g and 24.1 mg/g, respectively, in terms of N-acetyl neuraminic acid. The corresponding values for buffalo milk sample were 11.5 mg/g and 9.6 mg/g.

2. Amongst the milk proteins analysed, casein accounted for almost 80% of the total sialic acid present in milk and rest 20% was due to whey proteins and proteose-peptide.

3. Proteose-peptide fraction of milk exhibited an almost four-fold higher value of sialic acid compared to casein in milk.

4. Characterization of the sialic acid residue from proteose-peptide revealed the presence of NANA as established by ion-exchange and paper chromatography.

5. Proteose and proteose-peptide contained 2.78% and 2.30% hexose in case of cow milk and 1.09% and 0.80% for buffalo milk samples, respectively. The hexose fraction comprised glucose, galactose and glucosamine in these samples.

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